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Bleomycin-Induced Lung Injury in the Rat: Effects of the Platelet-Activating Factor (PAF) Receptor Antagonist BN 52021 and Platelet Depletion

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Bleomycin is a highly effective antitumor agent, but pulmonary toxicity, characterized by an acute inflammatory reaction and associated pulmonary edema, limits clinical use of the drug. Platelets and platelet-activating factor (PAF), a membrane-derived phospholipid, have been implicated in the mechanisms that can mediate pulmonary microvascular injury. We sought to investigate the role of PAF in bleomycin-induced lung injury in the rat, using the PAF receptor antagonist BN 52021; and the role of platelets though the use of an anti-platelet antibody. Lung injury was induced by intratracheal bleomycin (1.5 mg) and assessed by measurements of lung wet weight and total pulmonary extravascular albumin space (TPEAS). Bleomycin caused a significant increase in both indices after 48 hr, compared with control animals ($p < 0.05$). A single dose of BN 52021 (20 mg/kg orally) significantly reduced the bleomycin-induced increase in lung weight, but not the rise in TPEAS ($p > 0.05$). Increasing the dose of BN 52021 (20 mg/kg/12 hr, orally) had no additional effect. Reducing circulating platelet numbers by approximately 75% had no effect on either the increase in lung weight or TPEAS, observed 48 hr after bleomycin ($p > 0.05$). PAF may partially contribute to the acute inflammatory reaction seen after intratracheal bleomycin in rats.

Introduction

Bleomycin, an antibiotic derived from *Streptomyces verticillus*, is a highly effective antitumor agent that is widely used in the treatment of lymphoma and testicular and squamous cell tumors (*1*). Pulmonary toxicity is a prominent side effect of the drug, limiting the dose that may be used in clinical practice. In severely affected patients, a pneumonitis may develop similar to that of the adult respiratory distress syndrome (ARDS), characterized by increased vascular permeability, pulmonary edema, and refractory hypoxemia with a high associated mortality (*2–4*). Although several conditions predispose the lung to injury—including doses of bleomycin in excess of 450 mg, concomitant exposure to radiation, combination therapy with other agents, and impaired renal function—the mechanisms underlying the pulmonary toxicity remain unknown (*5*). Similarities in pathology and clinical presentation between bleomycin-induced lung injury and ARDS suggest a common etiology (*5,6*).

Bleomycin administered intratracheally in rats has been shown, in our laboratory and those of others, to produce histological changes similar to those seen in ARDS (*6*). These comprise an early inflammatory phase characterized by neutrophil influx and increased microvascular permeability, maximal after 72 hr (*7*), and followed by fibrosis that is maximal at 2 to 3 weeks (*8*). Many previous investigators have focused on the role of vasoactive substances and inflammatory cells in generating acute edematous lung injury (*9,10*). Platelet-activating factor (PAF) is a membrane-derived phospholipid that has been shown to mediate both the pulmonary microvascular injury and acute inflammatory changes that follow administering endotoxin to rats (*11*). In a previous study, we have also shown PAF to be a potent mediator of increased vascular permeability in the bronchial circulation of the guinea pig, an effect that can be abolished using a PAF-receptor antagonist, the

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ginkgolide mixture BN 52063 (*12*). The sequestration of platelets in the lungs of patients with ARDS, together with experimental evidence to suggest that platelets may directly damage alveolar capillary membranes and intereact with neutrophils to produce inflammatory mediators, has also led to speculation regarding their potential role in the generation of acute lung injury (*10*).

In the current study, we therefore investigated the effects of the ginkgolide PAF-receptor antagonist BN 52021, which is the active component of BN 52063, and of platelet depletion on the acute (inflammatory) phase of bleomycin-induced lung injury in the rat.

Methods

Male Lewis rats (175–225 g, Charles River, UK) were anesthetized with intramuscular Hypnorm (fentanyl citrate 0.315 mg/mL and fluanisone 10 mg/mL) at a dose of 0.5 to 1.0 mL/kg body weight, prior to each procedure.

Intratracheal Bleomycin

Animals received 1.5 mg of bleomycin in 0.3 mL of 0.9% saline by intratracheal instillation. Control animals received 0.3 mL of saline alone. Animals were sacrificed 48 hr after receiving bleomycin.

Administration of BN 52021

BN 52021, 20 mg/kg in 0.5% carboxymethyl cellulose (20 mg/mL), was administered by gavage at varying time intervals. Control animals received 0.5% carboxymethyl cellulose alone (1 mL/kg).

Assessment of Total Pulmonary Extravascular Albumin Space

Total pulmonary extravascular albumin space (TPEAS) was measured using a method modified from that of Wangensteen et al. (*13*) and described in full elsewhere (*6*). Twenty-four hours before being sacrificed, each animal received 1 to 2 μCi of ^{125}I-labeled human serum albumin (Amersham International Ltd., UK) IV via a tail vein, in a total volume of 0.5 mL of 0.9% saline. The animals were anesthetized and exsanguinated via the aorta 24 hr later. The pulmonary circulation was flushed free of blood by ligating a cannula within the right ventricle and perfusing with 12 mL of phosphate-buffered saline. The lungs were removed and dissected free of the main bronchi and blood vessels. They were gently blotted to remove excess surface fluid, weighed, and placed in a counting vial. Duplicates of 0.1 mL plasma were also counted in a gamma counter. Total pulmonary extravascular albumin space was calculated as the ratio of total lung radioactivity to that of 1 mL plasma.

Experimental Protocols

The effect of BN 52021 on bleomycin-induced lung injury was first assessed by administering a single dose of drug (n = 7) or the vehicle alone (n = 8) to rats 30 min prior to receiving bleomycin. A third control group received intratracheal saline (n = 5). All animals were sacrificed 48 hr later.

To test for a possible dose-dependent effect of BN 52021, in a second experiment animals received BN 52021 (n = 9) or the vehicle alone (n = 6) 30 min prior to the bleomycin and subsequently at 12-hr intervals up to 48 hr, when they were sacrificed. A third, control group received intratracheal saline (n = 3).

To assess the possible role of platelets in bleomycin-induced lung injury, in a third experiment animals received either 1.0 mL/kg rabbit serum by IP injection (n = 6), or 1.0 mL/kg of the rabbit anti-rat platelet antibody (IP, n = 6) 24 hr prior to the bleomycin. Full blood counts were measured 24 hr later, and the animal were sacrificed (as before) 48 hr after receiving the bleomycin.

Drugs and Chemicals

Drugs and chemicals were obtained from the following sources: Hypnorm from Janssen Pharmaceuticals (Oxford, UK); bleomycin from Lundbeck Pharmaceuticals (UK); BN 52021 was a kind gift from Dr. P. Braquet (Laboratories Henri Beaufour, Paris, France); and anti-platelet antibody a kind gift from Dr. K. Butler (Ciba Laboratories, West Sussex, UK).

Statistical Methods

Comparisons between groups were made using the Mann-Whitney U test. Values less than 0.05 were considered significant.

Results

Wet weight and TPEAS for animals in experiment 1 are shown in Figure 1. Administration of bleomycin caused a significant increase in lung weight and TPEAS in both groups of animals, compared with controls ($p < 0.05$). Administration of the PAF receptor antagonist BN 52021 caused a significant reduction in the increase in lung weight compared with vehicle (1.09 $\pm$ 0.10, mean $\pm$ SEM, vs. 1.64 $\pm$ 0.19 g, $p < 0.05$). The increase in TPEAS was attenuated by BN 52021, although this did not achieve statistical significance (0.31 $\pm$ 0.05 vs. 0.41 $\pm$ 0.05, $p < 0.05$).

Data from the second study, in which animals were given multiple doses of BN 52021, are shown in Figure 2. The increase in lung wet weight caused by bleomycin was significantly altered by BN 52021 (1.17 + 0.04 vs. 1.33 $\pm$ 0.05, $p < 0.05$). The bleomycin-induced increase in TPEAS was reduced with BN 52021, but, again, not significantly so (0.57 $\pm$ 0.01 vs. 0.41 $\pm$ 0.02, $p = 0.07$).

The results for the third study are shown in Figure 3. Platelets were significantly ($p < 0.05$) reduced in the antibody-treated group, compared with serum (mean count 252 $\times$ 109/L vs. 947 $\times$ 109/L). Neither hemoglobin nor white cell count were significantly altered. There was no significant difference between

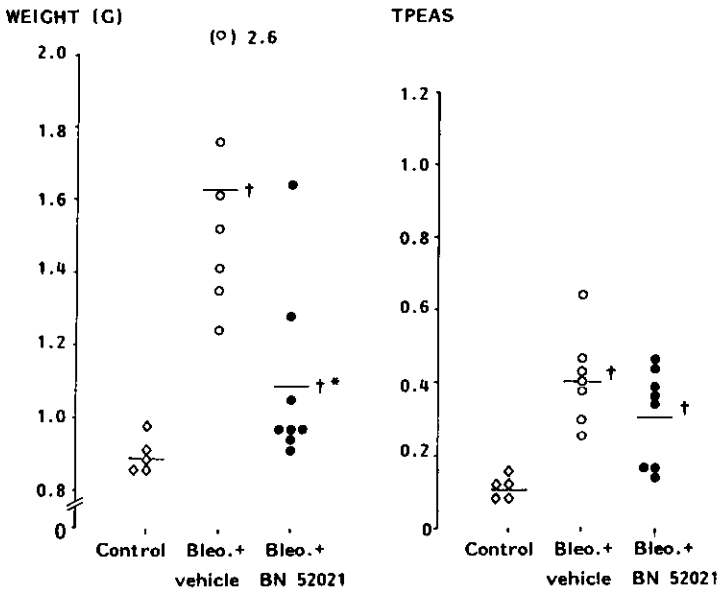

FIGURE 1. Changes in lung weight and total pulmonary extravascular albumin space (TPEAS) in rats treated with BN 52021 20 mg/kg or 0.5% carboxymethyl cellulose (vehicle) orally 30 min prior to receiving intratracheal bleomycin 1.5 mg or saline (control) and perfused 48 hr later. Horizontal bars indicate mean values. Dagger (†) indicates significant increase compared with control. Asterisk (*) indicates significant decrease compared with bleomycin plus vehicle.

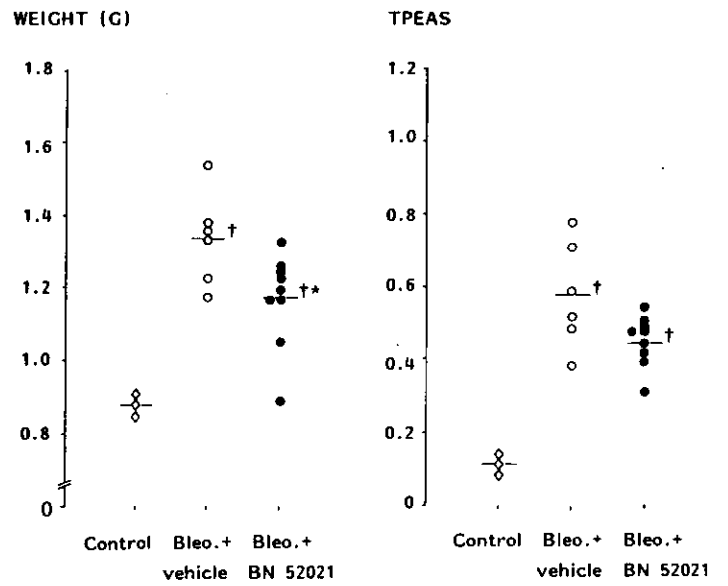

FIGURE 2. Changes in lung weight and total pulmonary extravascular albumin space (TPEAS) in rats treated with BN 52021 20 mg/kg or 0.5% carboxymethyl cellulose (vehicle) 30 min prior to intratracheal bleomycin 1.5 mg or saline (control) and at 12-hr intervals thereafter, and perfused after 48 hr. Horizontal bars indicate mean values. Dagger (†) indicates significant increase compared with control. Asterisk (*) indicates significant decrease compared with bleomycin plus vehicle.

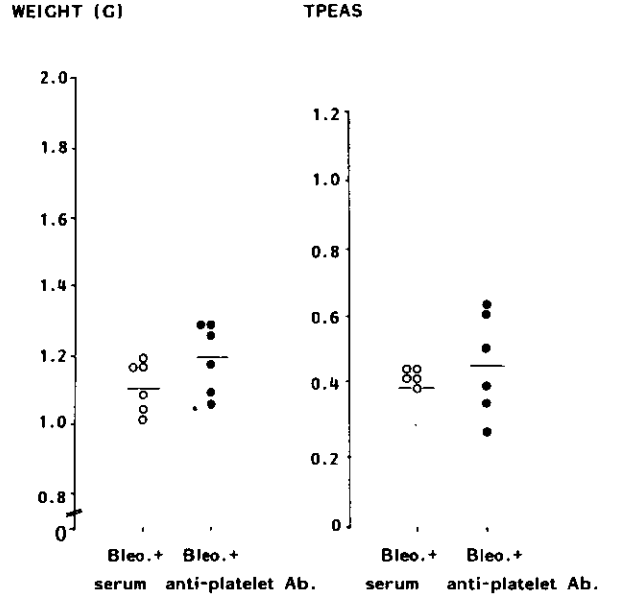

FIGURE 3. Changes in lung weight and TPEAS in animals treated with anti-platelet antibody or serum, 12 hr before treatment with BN 52021 20 mg/kg or 0.5% carboxymethyl cellulose (vehicle). Intratracheal bleomycin 1.5 mg was administered 30 min later. Horizontal bars indicate mean values.

the groups for either lung weight (1.10 ± 0.03 for vehicle vs. 1.19 ± 0.09 g for BN 52021 animals) or TPEAS (0.46 ± 0.06 vs. 0.40 ± 0.06).

Discussion

Any therapeutic intervention that might be developed by an understanding of the mechanisms underlying bleomycin-induced lung injury would considerably extend the clinical effectiveness of the drug. Our results suggest that PAF may be partially responsible for the acute inflammatory response seen after intratracheal administration of bleomycin in the rat, although only one of the measured indices, the lung weight, was affected by administration of the drug.

In previous studies changes in lung weight have been shown to correlate well with acute lung injury and subsequent fibrosis (*14*). Further attenuation of the effects of bleomycin was not achieved by increasing the dose of BN 52021. We previously showed that a smaller dose of a less potent, but related PAF receptor antagonist, BN 52063, is completely effective in abolishing the inflammatory effects of exogenously administered PAF, as indicated by changes in microvascular permeability in the bronchial circulation of the guinea pig (*12*). Interestingly, although increasing the total dose of BN 52021 administered in the second experiment did not further attenuate the increase in lung weight seen after bleomycin, the reduction in TPEAS almost achieved statistical significance ($p = 0.07$).

Wagensteen et al. (*13*) have shown that intratracheal bleomycin in the rat does not increase lung wet/dry weight ratio, suggesting that the increase in lung weight could be the result of inflammatory cell influx into the lungs. Previous work from our laboratory showed significant increases in the recovery of inflammatory cells such as neutrophils, lymphocytes, and monocytes in bronchoalveolar lavage fluid from rats that were treated with bleomycin (*6*). It is possible that the PAF antagonist BN 52021 inhibits the chemotaxis of neutrophils into the lungs since PAF possesses chemotactic effects for neutrophils *in vitro* (*15*).

Our measurement of albumin space, TPEAS, is not necessarily an index of vascular permeability to albumin as shown by Wagensteen et al. (*13*). Indeed, by using a new method for specifically assessing albumin permeability they found only a small significant increase after dosing with bleomycin, but TPEAS was approximately doubled, as we observed in our present study. It is possible that the increase in TPEAS is a reflection of a direct effect of bleomycin on the lung interstitial space that allows albumin to occupy more of the interstitial volume. Presumably, neither PAF nor platelets would effect this process. Thus, the two indices of lung injury we measured may reflect different pathological processes induced by bleomycin.

Despite the failure of BN 52021 to prevent pulmonary injury in the rat model, PAF may still be a partial mediator of bleomycin-induced lung injury in man. PAF is one of the most potent inflammatory mediators known to increase microvascular leakage in different vascular beds in several animal species, and it has many of the properties that can induce the pathophysiologic responses of acute lung injury. Pulmonary endothelial damage has been observed following the administration of PAF in several animal models, and it is indicated by increasing lymph flow, elevated lymphatic albumin and globulin content, and increased lung weight (*16*).

The IV administration of PAF has been shown to alter lung mechanics in rabbits and guinea pigs in a similar manner to that seen clinically in patients with bleomycin-induced pulmonary injury and ARDS (*17,18*). Endothelial damage accompanied by platelet and leukocyte plugs are also observed in the pulmonary capillaries of experimental animals after PAF administration (*19*). However, the release of PAF during induction of acute lung injury in experimental animals has yet to be demonstrated, possibly because of its short plasma half-life (*20*). The biological effect of PAF may be amplified by the presence of other mediators released from activated neutrophils and platelets. Such mediators include lysosomal enzymes, toxic oxygen radicals, thromboxane, and leukotrienes (*21,22*). It may be that a combination of antagonists to a variety of mediators may be required to achieve the further blockade of acute lung injury in this and other models. For example, in a previous study, we were able to only partially inhibit endotoxin-induced increases in airway microvascular permeability in guinea pigs using PAF receptor antagonists alone, suggesting that additional factors were involved (*23*).

Evidence suggests that platelets are a critical component in the generation of acute lung injury (*10*). In some species, the release of vasoactive inflammatory mediators is, at least, partially dependent upon the presence of platelets (*24*), which together with neutrophils release inflammatory mediators when activated by endothelial-derived mediators such as PAF and thromboxane. Aggregation of platelets and neutrophils in the lungs of experimental animals has been observed in models of acute lung injury (*21*).

We were unable to show that a reduction in platelet count caused any significant reduction in bleomycin-induced lung injury. This may have been due to an inadequate reduction in platelet numbers (approximately 75% inhibition), although the maximal effect of the antibody occurs 12 hr after administration, and the platelet counts (measured at 24 hr) may therefore not have reflected the period of maximum suppression.

The results of the present study suggest that PAF may be partly involved in mediating bleomycin-induced acute lung injury in the rat, perhaps by inhibiting the inflammatory cell recruitment. Nevertheless, it is increasingly clear from this and other investigations that no single pathway leads directly towards acute edematous lung injury. It is more likely that a large number of humoral factors, inflammatory cells, and mediators interact with other currently undefined agents to promote vascular endothelial cell damage and respiratory failure. Consequently, although further studies using newly available antagonists with greater potency (*25*) are indicated, these should possibly be carried out in combination with depletion of inflammatory cells.

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